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Sex Differences in the Ageing Rat: The Effect of Caffeine and Glucose, Alone and Combined, on Olfactory Memory

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Abbreviations

5'N	ecto-5'-nucleotidase
A1	A1 Adenosine receptor
A2A	A2A Adenosine receptor
AD	Alzheimer's disease
ADHD	Attention Deficit/Hyperactivity Disorder
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
AUC	Area under Curve
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
ecto-ATPase	ecto- Adenosine Triphosphase
IP	Intra-Peritoneal
IQ	Intelligence Quotient
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine
OC	Oral Contraceptive
PD	Parkinson's disease
SEAL	Sea, Air, Land (a division of navy combat)
SHR	Spontaneously Hypertensive Rat
TrkB	Tyrosine Kinase B
WM	Working Memory

Abstract

There is a reasonable body of evidence that caffeine can attenuate the memory deficits caused by Alzheimer's disease, ADHD and Parkinson's disease, as well as a variety of non-pathological causes such as ageing, fatigue, and substance use. Caffeine effects on memory in healthy individuals have been less consistent, and many studies have failed to isolate the effects of glucose (a known cognitive enhancer). In the current study, 119 middle-aged rats (61 males, 58 females) were tested in a novelty-related location preference paradigm which assessed the effects of caffeine (20mg/kg and 40mg/kg) and glucose (100mg/kg), alone and combined, on olfactory memory performance. The memory criterion was adjusted to accommodate a preference for familiarity rather than novelty, which was attributed to an attraction to self-produced odour. 12-month-old females displayed no memory retention, while 12-month-old males exhibited intact memory, and the administration of caffeine and/or glucose had no significant effect on memory performance. Sex differences were postulated to involve natural age-related decline of hormone levels. Additionally, ceiling effects may have limited the findings in males, as supported by a significant interaction between caffeine and glucose when assessed separately from saline. These findings warrant further research into the relationship between caffeine and glucose with methods that are more suited to intact subjects.

Introduction

Caffeine (1,3,7-trimethylxanthine) is the most widely consumed psychoactive substance in the world (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). As such, it has been studied extensively in terms of behavioural and cardiovascular effects, with benefits found for attention, vigilance, endurance, mood and reaction time (Nehlig, 2010; Smith, 2002). Caffeine exerts its effects by the antagonism of adenosine A_1 and A_{2A} receptors, which are present in almost all areas of the brain with the highest concentrations in the hippocampus, cerebral and cerebellar cortices (A_1), and dopamine-rich regions of the brain (A_{2A}; Fredholm et al., 1999). As well as attenuating the increase in drowsiness caused by adenosine, the antagonism of these receptors by caffeine results in an up-regulation of cholinergic and dopaminergic activity, which is thought to be the main mechanism of action for its effects on cognition (Hauber & Bareiß, 2001; Yonkov, 1985). Caffeine has been studied more recently for its possible memory-enhancing properties. However, findings have been mixed with outcomes dependent upon a variety of factors such as age, sex, cognitive functioning and time of administration. Additionally, many studies have failed to account for the presence of dietary and acute glucose, which is a known memory-enhancer (Riby, 2002). The current study will consider this previous research, and attempt to clarify the specific effects of caffeine on memory in both the presence and absence of glucose.

Caffeine

Lifetime Habitual Consumption

Several studies have examined the relationship between habitual caffeine use and cognitive performance with differing results. In cross-sectional studies, increased habitual caffeine consumption was associated with improved cognitive performance on several tasks, including delayed verbal recall (Jarvis, 1993). Additionally, these benefits were greater in

older participants compared to younger participants (although their overall performance was lower). Similarly, other researchers have found that higher caffeine consumption had no impact on immediate recall but was positively associated with delayed verbal recall (Hameleers et al., 2000). However, a follow-up of the same population sample 6 years later found no effects of caffeine on memory or any other cognitive measures (Van Boxtel, Schmitt, Bosma, & Jolles, 2003). A second longitudinal study included participants' IQ scores at age 11 and age 70, in order to control for early-life IQ being a predictor of later life cognitive functioning (Corley et al., 2010). They found that the positive association between caffeine and memory became non-significant once age 11 IQ and socioeconomic status were accounted for. The strong correlation between age 11 IQ and age 70 IQ ($r=0.66$, $p<.001$) supported the researchers' suggestion that the majority of the variance in elderly cognitive function was accounted for by childhood IQ. Unfortunately, while the researchers tested a variety of memory subtypes, they combined them into one score of overall 'memory', so may have lost some of the more nuanced variation in the data.

A limitation of lifetime consumption studies is selective attrition, which leads to a survivor bias whereby only higher functioning participants are included in the final analysis (e.g., due to poor health or participants' death). This is indicative of Corley et al. (2010) where the final sample had significantly higher age 11 IQ scores than the original cohort ($M_{sample} = 50.02 \pm 11.04$ vs $M_{cohort} = 36.74 \pm 16.10$). Additionally, researchers are forced to rely on self-reported averages of caffeine consumption, which are not always accurate. For example, the caffeine levels in instant coffee have been found to range from 21mg to 120mg per serving, while ground coffee ranged from 15mg to 254mg per serving (Food Standards Agency, 2004). Thirdly, the direct contributions of caffeine are difficult to quantify as there are a number of possible confounds over a person's life that are unable to be controlled for. Longitudinal studies may have the best chance of drawing conclusions as they have baseline

measures for comparison, but, as can be seen from Van Boxtel et al. (2003) and Corley et al. (2010), these baselines tend to invalidate any direct relationship between habitual caffeine and memory.

Age

Both chronic and acute caffeine have been reliably shown to attenuate age-related memory deficits arising from Alzheimer's disease (see below). Caffeine has also been studied in the context of natural age-related cognitive decline, with promising results.

Chronic administration

Animal studies assessing the effect of chronic caffeine on ageing memory have been reasonably positive. Although effects were not observed in mice (Arendash et al., 2009), chronic treatment with caffeine over 12 months offset the decline in object recognition memory seen in healthy ageing (18-months-old) rats (Costa, Botton, Mioranzza, Souza, & Porciúncula, 2008). Caffeine-treated rats not only outperformed their age-matched controls, their performance was comparable to that of healthy 6-month-olds. Costa et al. (2008) observed that these benefits occurred alongside a prevention of inflating levels of Brain-Derived Neurotrophic Factor (BDNF) and Tyrosine Kinase B (TrkB) in the hippocampus, which have been linked to memory processes and are found to increase significantly with age (considered below). These behavioural and cellular findings were supported by Sallaberry et al. (2013) who found that chronic caffeine improved both short- and long-term avoidance memory in middle-aged (12-months-old), rats as well as reducing hippocampal BDNF and TrkB levels, which were inflated in the placebo group.

Acute Administration

There is evidence that acute caffeine may have a more pronounced effect in older compared to younger participants. When all ages were analysed together, researchers found

no overall effect of caffeine on immediate recall (Hogervorst, Riedel, Schmitt, & Jolles, 1998). However, analysis by group revealed that middle-aged participants receiving caffeine recalled significantly more words than those receiving a placebo. Rees, Allen and Lader (1999) found that an acute dose of caffeine offset the decline in working memory performance for older participants. Acute caffeine also prevented the decline in immediate and delayed verbal memory seen over the course of a day in participants over 65 years old (Ryan, Hatfield, & Hofstetter, 2002). These results are consistent with observations in animal studies, where acute caffeine was shown to reverse the age-related disruption of both olfactory discrimination and social recognition memory in 12-month-old rats (Prediger, Batista, & Takahashi, 2005). These benefits were found to exist outside of an improvement in locomotion, meaning that caffeine was modulating memory performance, rather than overall energy levels.

Adenosine Function and Ageing

One theory as to why caffeine may have a different effect in older compared to younger subjects is due to the changes in adenosine function that occur with age. Research by Cunha, Constantio, Sebastião and Ribeiro (1995) found significant changes in adenosine receptor binding sites due to aging. Binding site density for adenosine A_1 receptors was decreased by 33% in the hippocampus and 60% in the cortex of aged rats (2 years) compared to young adult rats (6 weeks). Conversely, binding site density for A_{2A} receptors was increased by 32% in the hippocampus and 94% in the cerebral cortex of aged rats. This shift in balance between the inhibitory A_1 receptors and the excitatory A_{2A} receptors appears to promote an increase in neuronal excitability and neurotransmitter release in these areas of the brain associated with memory (Cunha et al., 1995).

Research on the hippocampus shows that aged rats have a decrease in the nucleotide Adenosine Triphosphate (ATP) and a corresponding decrease in ecto-ATPase – an enzyme

which sequentially hydrolyses extracellular ATP to Adenosine Diphosphate (ADP) and Adenosine Monophosphate (AMP; Heine, Braun, Heilbronn, & Zimmermann, 1999). However, there is an almost five-fold increase in the activity of ecto-5'-nucleotidase (5'N), which facilitates the final hydrolysis step from AMP to adenosine. Additionally, the removal of extracellular adenosine is decreased by almost 50% due to a reduction in adenosine transporter activity (Cunha, Constantio, & Ribeiro, 1997). This significant increase in 5'N activity, coupled with the decreased adenosine transporter activity, results in increased transient extracellular adenosine in aged rats. Therefore, although less ATP is released with ageing, it may be more efficiently converted into adenosine (Cunha, 2001).

The activation of adenosine receptors has many effects on neuropeptide and neurotransmitter systems in the CNS. As mentioned above, Costa et al. (2008) linked the improvement of memory by caffeine to the prevention of increasing TrkB receptor levels in aged rats (the primary BDNF receptor). Previous research has shown that TrkB receptors are activated by adenosine through A_{2A} receptors (Lee & Chao, 2001), and that presynaptic release of adenosine via A_{2A} receptor activation triggers the action of BDNF, which facilitates synaptic transmission in the hippocampus (Diógenes, Fernandes, Sebastião, & Ribeiro, 2004). Additionally, BDNF-mediated effects in the hippocampus and striatum are eliminated by the removal of adenosine A_{2A} receptors (Tebano et al., 2008). Taken together, these findings further indicate a role for adenosine in the ageing brain; whereby aged rats have increased extracellular adenosine and increased A_{2A} receptors, which are known to increase BDNF function, and that chronic administration of caffeine (by its antagonism of A_{2A} receptors) prevents the over-inflation of BDNF in the hippocampus. This cellular research is mirrored behaviourally by the retention of recognition and avoidance memory in aged rats alongside the reduction of BDNF after chronic caffeine administration (Costa et al., 2008; Sallaberry et al., 2013).

Sex

Sex and memory

Sex differences are important to consider due to the relationship between hormones and cognition, and hormones and caffeine. There are a number of studies demonstrating the effect of sex on learning and memory in humans. Females show superior performance to males on verbal memory tasks including word-generation, word list recall, story recall, and verbal recognition (for a review, see Andreano & Cahill, 2009). Males generally perform better than females on spatial memory tests, although females have significantly better object location memory, suggesting that the addition of internal verbal memory to spatial information modulates performance (Andreano & Cahill, 2009). Indeed, as object location tasks become more purely spatial, females lose their performance advantage (James & Kimura, 1997). Women also have an advantage in autobiographical memory, displaying more accurate and more specific recall of events (Pohl, Bender, & Lachmenn, 2005; Ross & Holmberg, 1992). Because sex differences in verbal memory appear in childhood it is believed that they are caused by differences in brain organisation rather than the influence of sex hormones, as shown by the fact that women continue to perform better than men when they are matched for estradiol levels (Yonker, Eriksson, Nilsson, & Herlitz, 2003).

As well as between-sex differences, there is also evidence of differences in cognitive performance during different phases of the menstrual cycle, where peak levels of estrogen have been shown to have task-specific effects, facilitating the process of automatisation on highly practised tasks while inhibiting performance on perceptual restructuring tasks (Broverman et al., 1981). During ovulation in normally-cycling women (peak estrogen) there was significant impairment in short-term recall of semantically similar words and, conversely, a trend towards facilitation in recall of acoustically similar words (Hartley, Lyons, & Dunne, 1987). When combined, a significant interaction between the type of words

(task) and cycle phase on memory performance indicates that ovulation had opposite effects on these different memory tasks. Postma, Winkel, Tuiten and van Honk (1999) found that males had superior accuracy to females on a spatial memory task, while, within the female group, performance was significantly better during the non-menstrual phase compared to menstruation. These results suggest that sex hormones have a more complex within-sex role in verbal and spatial memory than was previously thought.

There is also evidence of an increased glucocorticoid cortisol release during the luteal phase compared to the follicular phase, which may facilitate consolidation of stressful or traumatic memories (Burgess & Handa, 1992). Andreano, Arjomandi and Cahill (2008) found that women had better memory for stressful events when the stressful event occurred during the mid-luteal phase (coinciding with high salivary cortisol levels) but not during any other time. Women also had a 3.64 times higher likelihood of experiencing acute flashback memories when trauma occurred to them during their luteal phase (Bryant et al., 2011). Women in the luteal phase at encoding of emotional stories showed enhanced memory for periphery detail rather than core detail, while women who were in the follicular phase during encoding showed no enhanced recall in any condition (Nielsen, Ahmed, & Cahill, 2013). These differences were not attributable to arousal or attentional differences, and suggest that menstrual cycle phase may play a role in long-term emotional memory recall.

Sex and caffeine

Sex hormones such as estrogen have a marked effect on caffeine metabolism and elimination. The half-life of caffeine was significantly prolonged in those taking chronic low-dose estrogen (<50μg) in oral contraceptives (OCs) compared to matched controls (M_{OCs} = 7.88 hours, $M_{control}$ = 5.37 hours; Abernathy & Todd, 1985). While there were no differences in peak plasma concentrations of caffeine, the time to reach peak concentration was delayed in those using OCs. A within-subjects design revealed that the administration of OCs

increased the mean residence time (MRT) of caffeine in women by almost two-fold compared to their own baseline scores (M_{OCs} = 11.1 hours, $M_{Baseline}$ = 5.8 hours; Reitveld, Broekman, Houben, Eskes, & van Rossum, 1984). The same study found that those taking OCs had a higher concentration of caffeine in blood plasma over time. As well as responding to hormone administration, caffeine elimination fluctuates naturally across the menstrual cycle, with a significantly slower clearance of caffeine during the luteal phase compared to the follicular phase (Lane, Steege, Rupp, & Kuhn, 1992).

Taken together, these results suggest that those with higher levels of estrogen due to the menstrual phase or the use of oral contraceptives have a decreased clearance speed of caffeine, meaning that caffeine levels tend to accumulate more during daily consumption. While this is not likely to reach levels of toxicity, it may have clinical implications for testing paradigms and practical implications with regard to caffeine elimination and sleep.

Sex, memory, and caffeine

Females are underrepresented in studies of caffeine and memory, despite recommendations from the Institute of Medicine that sex should be included as a variable in all biomedical and health-related research (Wizemann & Pardue, 2001). Some sex differences have been reported in habitual consumption studies, where caffeine had a facilitatory effect on the verbal memory in women, but not in men (Ritchie et al., 2007). A separate study revealed that although male participants drank more coffee than women across their lifespan, higher habitual consumption was not associated with any memory improvements in men, while women with higher caffeine consumption had improved performance on five out of six verbal and visual memory tasks (Johnson-Kozlow, Kritz-Silverstein, Barrett-Connor, & Morton, 2002).

Acute studies have been extremely limited and fail to provide a conclusive understanding of the role of sex in the relationship between caffeine and memory. One early

study found that caffeine had no performance effects in males, and no effect in females at a low task-load, but impaired female word recall compared to placebo during a high task-load (Erikson et al., 1985). Arnold, Petros, Beckwith, Coons and Gorman (1987) replicated the study with a larger male cohort, and excluded females who used oral contraceptives (as well as testing them during the first five days of the menstrual cycle, when estrogen is at its lowest). The results were vastly different; for females, caffeine facilitated recall after practice with the task, while males showed a trend-significant interaction between dose and task-load ($p=.058$), whereby low-dose caffeine impaired performance during a high task-load, while high-dose caffeine improved performance at a low task-load. These complex interactions between sex, dose, and task-load confirm the importance of including both male and female participants in studies of caffeine and memory. Additionally, the differing results between these studies demonstrate the importance of controlling for variables such as OCs, which can have a significant impact on memory performance in females.

Reversing Impairment

Alzheimer's Disease

Alzheimer's disease (AD) is an age-related disorder characterised by the build-up of ß-amyloid plaques and neurofibrillary tangles leading to a progressive loss of memory, reasoning and language (Selkoe, 2011). A_{2A} receptor antagonists are effective at reducing neurotoxicity caused by ß-amyloid protein in the hippocampus, and, thus, caffeine has been studied as a prospective treatment option (Cunha, 2005). There is evidence for an inverse relationship between lifetime habitual caffeine intake and instances of Alzheimer's disease, whereby higher caffeine consumption was associated with lower instances of AD, and vice versa (Maia & de Mendonça, 2002). In animal studies, caffeine treatment of 1.5mg/day over four months was found to protect neurons against ß-amyloid toxicity in a transgenic mouse model of Alzheimer's disease, which prevented the development of cognitive impairments in

working, retention, and spatial memory (Arendash et al., 2006). To determine whether these effects could be seen after cognitive impairment had already developed, Arendash and colleagues administered chronic caffeine (1.5mg/day for 4-5 weeks) to aged transgenic mice once they had already displayed spatial and working memory deficits. Transgenic mice receiving caffeine demonstrated significantly better working memory than transgenic controls, and their performance was equal to healthy controls at the last block of testing, suggesting that these memory deficits were able to be reversed after impairment was already established (Arendash et al., 2009). Chu et al. (2012) also found that a chronic two month administration of both caffeine and crude caffeine reversed the spatial learning and memory deficits seen in transgenic mice. However, in a delayed recall probe task one day later, mice who received caffeine were unable to significantly outperform controls, whereas mice who receive crude caffeine displayed improvements in long-term memory. Researchers suggested that these differences may be due to the variety of bio-active compounds found in crude caffeine which are not present in pure caffeine, such as catechin, a natural antioxidant. As well as chronic doses, acute caffeine (30mg/kg) has been found to reverse the negative effects of ß-amyloid on aversion memory in mice, as did a sub-chronic dose over 4 days (Dall'Igna et al., 2007).

Attention Deficit/Hyperactivity Disorder

Attention deficit/ hyperactivity disorder (ADHD) presents as a set of pervasive behavioural symptoms beginning in childhood, and is thought to encompass a variety of executive dysfunctions including verbal and spatial working memory (Castellanos, Sonuga-Barke, Milham, & Tannock, 2006). The use of caffeine as a possible treatment strategy stems from early epidemiological studies suggesting reduced incidence of ADHD in countries with higher per capita caffeine consumption (Dalby, 1985; Schnackenberg, 1975). Although some studies have explored the effects of caffeine on general cognitive symptoms, its specific

effect on memory is yet to be determined in human trials. However, the use of the Spontaneously Hypertensive Rat (SHR) as an animal model of ADHD has provided some positive results. Acute pre-training caffeine significantly improved memory for a previously-encountered juvenile (Prediger, Fernandes, & Takahashi, 2005), and also increased spatial memory in SHRs (Prediger, Pamplona, Fernandes, & Takahashi, 2005). However, these effects were not found to carry over to a delayed session 48 hours later, and post-training or pre-test caffeine administration had no effect on test performance in SHRs, suggesting that benefits may be due to the specific timing of administration relating to different stages of memory processing rather than a global effect (Prediger, Pamplona, et al., 2005). Animal research has also revealed neuroprotective effects, whereby chronic pre-pubertal caffeine improved the object recognition impairment normally seen in SHRs, while having negative effects on the control Wistar rats (Pires, Pamplona, Pandolfo, Prediger, & Takahashi, 2010). Difficulties arise with ADHD research due to the disorder presenting in childhood, so treatment doses must be altered to match the pharmacokinetic profile of caffeine in children. Nevertheless, the preliminary positive results in animal trials for spatial, social, and object recognition memory as well as the possible neuroprotective effects of habitual caffeine on ADHD warrant further investigation into this relationship.

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder characterised by impairments in motor function and cognitive processes (including working and episodic memory) resulting from a depletion of dopamine in the substantia nigra of the brain (Prediger, 2010). Longitudinal studies have provided some insight into possible neuroprotective effects of caffeine on PD. Habitual caffeine has been linked to a decreased risk of PD for men, however, women are thought to have a U-shape response where a moderate caffeine intake is associated with the lowest risk of PD (Ascherio et al., 2001). This

it attributed to an interaction with postmenopausal estrogen levels, where high caffeine consumption increased the risk of PD in women who used estrogen replacement therapy, but reduced PD risk in women who did not (Ascherio et al., 2003). These findings suggest that the positive effects of caffeine on the risk for PD may be negated or even reversed by the use of estrogen replacement therapy.

Animal models of PD involve lesions via an injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) to the substantia nigra pars compacta of the brain. Caffeine has been found to protect dopamine neuron toxicity when administered up to two hours before and two hours after an injection of MPTP (Xu, Xu, Chen, & Schwarzschild, 2010). Behavioural evidence supports this effect, with acute pre-training caffeine offsetting the learning and long-term avoidance memory deficit in MPTP lesioned rats (Gevaerd, Takahashi, Silveira, & Da Cunha, 2001). Acute caffeine also reversed social memory deficits in an alternative animal model of PD using injections of reserpine (which depletes dopamine in the brain), and was comparable to a dopamine D_2 agonist at increasing recognition ability (Prediger, Da Cunha, & Takahashi, 2005).

Non-Pathological Impairment

Caffeine has been found to prevent and reverse memory decline due to ethanol ingestion. Participants receiving caffeine and ethanol maintained verbal memory ability at placebo levels while those receiving ethanol-only were significantly impaired (Drake, Rohers, Turner, Scofield & Roth, 2003). Animal studies have shown that acute caffeine administration either 20 minutes pre-acquisition or one hour post-ethanol negated the effects of ethanol on olfactory memory in rats, facilitating memory at both the encoding and the recall stages (Spinetta et al., 2008). Caffeine has also been shown to prevent and reverse memory impairments caused by other substances, such as scopolamine (Botton et al., 2010; Riedel et al., 1995).

In studies of fatigue (caused by sleep deprivation), caffeine had no effect on memory accuracy in participants tested at 3am (Lorist, Snel, Kok, & Mulder, 1996), but improved short-term memory in individuals tested at 6am (Linde, 1994). Under more extreme conditions (after three days of sleep deprivation), SEAL trainees receiving caffeine completed a motor learning and memory task more quickly and more accurately than their non-caffeinated peers (Lieberman, 2003). As well as mental fatigue, acute caffeine was found to increase short- and long-term memory after strenuous exercise in endurance athletes (Hogervorst, Riedel, Kovacs, Brouns, & Jolles, 2007). Therefore, while caffeine attenuates the memory deficits caused by extreme fatigue, these benefits are less pronounced in mildly fatigued individuals. As well as acute doses, chronic caffeine has been found to prevent impairments in long- and short-term memory caused by 24-hour sleep deprivation in rodents (Alhaider, Aleisa, Tran, & Alkadhi, 2011; Alhaider, Aleisa, Tran, Alzoubi, & Alkadhi, 2010).

Enhancement in Healthy Subjects

While caffeine has been shown to improve memory in subjects with some level of natural or induced impairment, enhancement effects in fully functioning subjects have been rather inconsistent. A few studies have reported that caffeine enhanced memory in all conditions (Capek & Guenther, 2009; Roussinov & Yonkov, 1976; Wright et al., 2013), however many have found no beneficial effect of caffeine on memory retention (Addicott & Laurienti, 2009; Haskell, Kennedy, Wesnes, & Scholey, 2005; Herz, 1999; Hewlett & Smith, 2006; Koppelstaetter et al., 2008; Rogers & Dernoncourt, 1998; Smith, Brice, Nash, Rich, & Nutt, 2003; Smith, Clark, & Gallagher, 1999; Smith, Sturgess, & Gallagher, 1999). Of these studies, several suggested that while caffeine was not seen to directly improve memory accuracy, it facilitated task performance by improving other aspects of cognition. For example, in studies without improvements in memory, caffeine was found to improve sustained attention (Rogers & Dernoncourt, 1998), the speed of encoding new information

(Smith et al., 2003; Smith, Clark, & Gallagher, 1999), the speed of memory retrieval (Addicott & Laurienti, 2009; Hewlett & Smith, 2006; Smith, Sturgess, & Gallagher, 1999) and reaction time (Haskell et al., 2005). These findings indicate that caffeine may enable participants to complete tasks at a faster rate without compromising their accuracy. Alternatively, they are able to complete more trials within a given time-frame. Thus, although memory accuracy may not have been directly enhanced in these studies, overall memory performance was still facilitated. These effects (e.g., speed of encoding) have also been observed in the presence of enhanced memory accuracy (Roussinov & Yonkov, 1976).

Many studies on healthy subjects contain mixed accuracy results, whereby some but not all conditions were enhanced by caffeine (Angelucci et al., 1999; Borota et al., 2014; Cestari & Castellano, 1996; Heatherley, Hayward, Seers, & Rogers, 2005; Warburton, 1995). Aside from variability of dosage, a consistent factor in the presence or absence of caffeine effects was the time of administration. There is evidence that caffeine improves memory at encoding and retention stages but not at acquisition, as demonstrated by significant memory improvements when caffeine was administered post-training and pre-testing, but not pre-training (Angelucci, Cesário, Hiroi, Rosalen, & Cunha, 2002; Angelucci et al., 1999). The most consistent evidence is for an effect of caffeine administered immediately post-training, which enhanced memory in the above studies as well as in cases where pre-test caffeine and delayed post-training caffeine (+2 hours) were non-significant (Borota et al., 2014; Cestari & Castellano, 1996).

Some ‘human factors’ may also account for variability in the results from healthy individuals. Smillie and Gökçen (2010) found no main effect of acute caffeine on working memory (WM), but there was a significant interaction of caffeine and extraversion on overall WM performance, meaning that the effect of caffeine on working memory was entirely dependent on extraversion. Smith (2013) also found no main effect of acute caffeine or

extraversion on WM, but also found a significant interaction, whereby extraverts performed better than introverts on these tasks after receiving caffeine, but, conversely, introverts performed better than extraverts after receiving a placebo (decaffeinated coffee). Smillie and Gökçen (2010) suggested that caffeine, as an indirect dopamine agonist (through its antagonism of A_{2A} receptors located in dopamine-rich areas of the brain), interacts with personality traits such as extraversion through its effects on the prefrontal cortex. This theory is consistent with studies showing that working memory performance was impaired by dopamine antagonists in extraverts, but not in introverts (Wacker, Chavanon, & Stemmler, 2006).

Expectancy effects may also contribute to the variability in results between studies. There is strong evidence for the role of expectancy in psychomotor tasks, where participants who were told that they had been given strongly caffeinated coffee (when, in fact, they received decaffeinated coffee) performed better when they expected caffeine to enhance their abilities, and performed worse when they expected caffeine to impair their abilities (Fillmore & Vogel-Sprott, 1992). Similarly, caffeine enhanced performance on a sustained attention task but only when participants had been accurately told they were receiving it (participants who received caffeine, but were told that they received placebo, performed at placebo level; Elliman, Ash, & Green, 2010). Unfortunately, investigations into caffeine expectancy effects in memory tasks are lacking, with a wide search revealing only two studies analysing memory separately from other cognitive measures. Kelemen and Creeley (2001) found a trend-significant relationship between expectation, caffeine and memory, whereby caffeine improved memory but only in those who expected it to. Conversely, Oei and Hartley (2005) found no effect of caffeine or expectancy on verbal memory. This is possibly due to methodological differences where, instead of the experimenter informing participants whether caffeine would enhance or impair their performance, participants' own pre-existing beliefs about caffeine efficacy were obtained via self-report. In any case, the strong effect of

caffeine expectancy on cognitive performance in other tasks warrants consideration in human memory studies. Taken together, these findings demonstrate that the addition of more parameters can reveal significant effects and interactions where there were previously none; an important consideration for human studies in cognition.

Caffeine Summary

There is a reasonable body of evidence that caffeine can attenuate age-related cognitive decline, as supported by both behavioural and biobehavioural studies. Additionally, there is evidence that caffeine can prevent and reverse the memory deficits caused by Alzheimer's disease, ADHD and Parkinson's disease, as well as a variety of non-pathological causes such as ageing, fatigue, and substance use. Caffeine effects on healthy individuals have been less positive, with human factors such as personality traits and expectancy moderating results. However, post-training administration appears to have the most consistent effects in healthy subjects, where it is thought to improve memory at the consolidation stage, enhancing performance in subsequent trials. Research looking at the effect of sex on caffeine and memory has been limited, but there is evidence of complex interactions including task load and dose. Several problems in methodology limit the interpretation of these results. In addition to those outlined previously, perhaps the most prevalent concern is the failure of many studies to isolate glucose as a potential confounding factor. The memory-enhancing properties of glucose are considered below.

Glucose

Glucose is as fundamental molecule used by all living cells as a source of energy (Purves, Sadava, Orians, & Heller, 2001). There is a reasonable body of evidence that glucose consumed just after a learning experience can strengthen memory (or reduce forgetting) for that experience. Consistent memory improvements have been observed in subjects with impaired functioning, notably with Alzheimer's disease (Watson & Craft,

2004), and non-pathological ageing (Hall, Gonder-Frederick, Chewning, Silveira, & Gold, 1989). Glucose has also been shown to improve memory for healthy individuals in a range of experimental situations (for a meta-analysis, see Riby, 2002). Messier (2004) has suggested that the memory improving action of glucose is an indicator of pre-existing memory deficits, and that the results seen in 'healthy individuals' are actually a sign that those individuals have impaired glucose regulation, which is attenuated by acute glucose administration. This theory may account for some inconsistencies observed in studies on healthy individuals (Azari, 1991; Benton & Owens, 1993; Foster, Lidder, & Sünram, 1998; Winder & Borrill, 1998).

In a series of experiments on mice, poor gluco-regulation was associated with memory improvement by glucose, while glucose had no beneficial effect on memory in subjects with good gluco-regulation (Messier, 1998; Messier & Destrade, 1988). Impaired gluco-regulation is associated with impairment in tasks that rely on normal functioning of the hippocampus (Winocur, 1991), and an acute administration of glucose alleviates these deficits (Winocur, 1995). Similar results are found in human studies, where students with poorer glucose regulation performed worse on tests of verbal memory (Awad, Gagnon, Desrochers, Tsiakas, & Messier, 2002). Additionally, glucose improved memory performance in students with poorer glucose regulation but it did not affect students with better glucose regulation (Messier, Pierre, Desrochers, & Gravel, 1998).

The effects of glucose on memory are often much less prominent in young participants than in older ones (e.g., Hall et al., 1989). Messier (2004) suggests that this is because, typically, older people tend to have worse gluco-regulation than younger people. A study of age-related cognitive decline found that individuals with worse gluco-regulation displayed memory impairments and a reduction in hippocampal volume, a brain area that is crucial for learning and memory (Convit, Wolf, Tarshish, & de Leon, 2003). Research suggests that both prolonged hyperglycemia (Greenwood, Kaplan, Hebblethwaite, & Jenkins,

2003; Kaplan, Greenwood, Winocur, & Wolever, 2000) and prolonged hypoglycaemia (Convit et al., 2003; McNay & Gold, 2001) lead to impairment in memory performance and are associated with a decrease in both the structure and the function of the hippocampus (Messier, 2004).

Whether or not the beneficial effects of glucose reflect its action as a cognitive enhancer or simply a cognitive normaliser, the evidence for its facilitation of memory is robust. As such, it is an important variable to account for in studies investigating caffeine effects.

Caffeine and Glucose

There has been limited research investigating a possible additive effect of caffeine and glucose on memory. Current published studies are promising, but some procedural issues limit the scope of their findings. Research into caffeine and glucose is often conducted in the context of an 'energy drink' (ED), which has provided mixed results. EDs have been shown to improve attention and reasoning compared to placebo, but not verbal or spatial memory (Warburton, Bersellini, & Sweeney, 2001). EDs were also found to improve working memory accuracy on an attention task, but neither glucose, caffeine or EDs improved immediate or delayed word recall (Smit, Cotton, Hughes, & Rogers, 2004). Conversely, Scholey and Kennedy (2004) found that EDs had no effect on working memory, but significantly improved delayed recall as well as a combined 'global memory' score above what would be predicted from each constituent alone. However, none of these studies included a glucose+caffeine condition, only full EDs which also contained other substances including taurine, ginseng, ginkgo biloba and niacin, all of which have been implicated in memory performance (El Idrissi, 2008; Lee et al., 2013; Loriaux, Deijen, Orlebeke, & De Swart, 1985; Singh, 2004). In a separate study, Kennedy and Scholey (2004) found that glucose+caffeine significantly improved attention and WM performance compared to

placebo, however, glucose and caffeine were not administered separately so their additive effects were unable to be quantified.

The few studies that have assessed glucose and caffeine together and separately have found evidence of a synergistic effect on memory. Adan and Serra-Grabulosa (2010) found that while caffeine and glucose were unable to improve memory individually, a combination of the two reduced reaction time and increased both immediate and delayed verbal memory. Giles et al. (2012) found an effect of caffeine but not glucose on working memory, while the combination of caffeine+glucose increased WM scores above those achieved by caffeine alone. An fMRI study provided insight into possible drug effects even when no behavioural effects were found. While neither glucose, caffeine, nor glucose+caffeine had an effect on WM accuracy, blood oxygen analysis revealed changes in brain function for the glucose+caffeine group, whereby there was decreased activity in areas of the brain associated with attention and memory processes (Serra-Grabulosa, Adan, Falcón, & Bargalló, 2010). Researchers interpreted this as a possible synergistic effect whereby a combination of caffeine and glucose increased the efficiency of these processes, meaning less energy was required to achieve the same behavioural results. These findings may have an impact on the interpretation of previously non-significant results, as they indicate a facilitatory effect of caffeine+glucose on memory processes even when this effect is not observed behaviourally. Although these results are promising, some limitations are apparent. As all studies were conducted on human participants, researchers have listed human factors as potential confounds, such as expectancy, concentration and ‘mental effort’. In those studies that report synergistic effects, researchers note that the use of only one dose of each substance limits the interpretation of these relationships. It is clear that more research is required in this area to determine whether caffeine and glucose have a synergistic effect on memory retention or whether results are comparable to each substance’s performance alone.

Summary Rationale and Aims

At present, there are no published animal studies assessing a synergistic effect of caffeine and glucose on memory. Animal studies are important as they eliminate many limitations of human studies, including expectancy effects, interactions with personality traits, mental effort, concentration, habitual consumption (of caffeine, alcohol, drugs) and dietary differences. Significant sex differences have been found in relation to memory performance and caffeine metabolism separately, however many studies fail to isolate or even include sex as a factor in studies of caffeine and memory. The current study includes male and female subjects to ensure that this variable is accounted for. Additionally, a limitation of previous synergy studies is the use of a single dose. Therefore, the current study uses 20mg/kg and 40mg/kg caffeine (with and without glucose) to determine if there are any changes in the nature of this relationship between doses. Caffeine research has often assessed memory within the paradigm of an attention and WM task, which can make extrapolating the specific effects of caffeine on memory difficult (as its beneficial effects on attention are well established). Therefore, the current study employs a single, simple memory test that does not require any conditioned behaviours, and, instead, utilises rats' natural curiosity for novelty. Although the ability of caffeine and glucose to act as cognitive enhancers in subjects already functioning at 'peak' levels is uncertain, their memory improvements in under-performing subjects are well established. The current study assesses the effects of caffeine and glucose, alone and combined, on memory performance in middle-aged subjects, which may have implications for the enhancement of memory in natural, age-related cognitive decline, as well as pathological impairment in disorders such as Alzheimer's disease. The major aim of the present study is to determine whether or not both substances administered together will result in a stronger effect than either one on its own.

Method

Subjects

Initial subjects were 120 experimentally naïve PVG/c hooded rats (60 male, 60 female) from the Animal Facility, Department of Psychology, University of Canterbury, New Zealand. One male and two females developed tumours and were euthanised prior to testing. Due to the age of the rats, it was impractical to source one male and two females of the same age group so two available experimentally naïve males were used as replacements. Thus, the saline + saline group had one less subject (N=19) compared to the other groups (N=20). Group distributions are shown in Table 1. Subjects tested were 119 PVG/c rats (62 males, 57 females).

Group	Abbreviations	Males	Females	N
Saline+ saline	Saline	10	9	19
Glucose 100mg/kg+ saline	Glucose	11	9	20
Caffeine 20mg/kg + saline	C20	10	10	20
Caffeine 40mg/kg + saline	C40	10	10	20
Caffeine 20mg/kg + glucose 100mg/kg	C20+Gluc	10	10	20
Caffeine 40mg/kg + glucose 100mg/kg	C40+Gluc	10	10	20
Total		**61**	**58**	**119**

Table 1. Group abbreviations and distributions for testing

On post-natal day 30, the pups were weaned and housed in standard housing conditions, comprising opaque plastic cages (550 mm long × 360 mm wide × 220 mm high) with a stainless steel wire lid. All subjects had access to food pellets and water ad libitum throughout the study. The holding room was controlled for temperature (22°C ± 1°C) and humidity (48% ± 10%) on a 12-hour light/dark cycle (lights on at 0800 hours). Subjects were

housed in same-sex groups of four per cage, however, following the euthanising of the rats with tumours, three cages were left with three rats per cage and another cage was introduced to house the two replacement rats. All subjects were approximately 12 months old at the time of testing, and weighed between 176.5g - 304.3g (females) and 373.6g - 515.9g (males). All tests were carried out between 0900 and 1700 hours. Animals were treated in accordance with the New Zealand Animal Welfare Act, 1999, and all procedures regarding subjects, housing, and treatment were approved by the Animal Ethics Committee of the University of Canterbury.

Drug Administration and Rationale for Doses

Caffeine (2g) was dissolved in saline (100ml) to produce a solution of 40mg/kg, and 1ml of saline was added to every 1ml of the 40mg/kg solution to produce a 20mg/kg solution. D-Glucose (5g) was dissolved in saline (100ml) to produce a solution of 100mg/kg. Each rat received two inter-trial (i.e., post-consolidation) intra-peritoneal (IP) injections in a volume of 2ml/kg relevant to the group it had been assigned to; saline + saline, glucose 100mg/kg + saline, caffeine 20mg/kg + saline, caffeine 40mg/kg + saline, caffeine 20mg/kg + glucose 100mg/kg, or caffeine 40mg/kg + glucose 100mg/kg. These doses have been shown to be behaviourally effective in rats without inducing any toxic reactions (e.g., Hughes & Greig, 1976; Hughes, 2006). Two injections were administered for practical reasons; mixing the caffeine and glucose together and injecting the mixture would require an unreasonably large volume/injection especially with male rats, resulting in the use of larger syringes than those (1ml/kg) normally used for administering psychotropic drugs to rats of this strain (PVG/c). Splitting the interaction dose into its constituents allowed for the most accurate administration, and injecting the remaining subjects with an additional dose of saline ensured that the procedure was consistent among all animals. Rats received one IP injection in each side to minimise discomfort.

Consideration was also given to the timing of drug administration. To test memory processes, drugs can be administered pre-acquisition (learning and consolidation), post-acquisition (consolidation), and pre-testing (recall and consolidation, depending on the time-frame). There has been concern expressed over pre-acquisition and pre-testing methods as they do not rule out any acute pharmacological effect on attentional, perceptual, and other cognitive processes that may skew memory results (Hughes, 2006; Messier, 2004). Post-acquisition administration allows for the subject to experience both acquisition and testing scenarios out of a drug-state, and has been found to be comparable to pre-acquisition administration for glucose (Manning, Parsons, & Gold, 1992), as well as producing the most consistent effects for caffeine (see introduction). Thus, in the current study, IP injections were administered immediately after acquisition, and subjects were returned to their home cage pending a retention trial twenty-four hours later.

Apparatus and Behavioural Measures

This behavioural paradigm has previously been used as an effective measure of drug-modified memory that does not depend on conditioning or deprivation (Hughes, 2006). In free tests of exploration, novelty-related location preferences are well established, whereby rodents confined to a familiar area before being allowed access to a novel area show a significant preference for novelty (for a review, see Hughes, 1997). Thus, in the current paradigm, rats' memory for the novel and familiar halves of an exploration box is determined by the observation of a significant preference (more time spent) for the more novel chamber.

Exploration Box

The behavioural test was simple to administer and did not require prior training of the animals or any form of deprivation or shock. Subjects were tested in one of eight exploration boxes in a room lit by overhead fluorescent lighting. Boxes were positioned side-by-side with

an approximate 15cm gap between them. Each box was made up of two chambers (20cm x 20cm x 20cm) connected by a doorway in the wall between them (8cm x 20cm). During acquisition trials the doorway was blocked off by a removable guillotine slide (Figure 1A), which was removed for the retention trial (Figure 1B). The exploration box and guillotine door were constructed out of wood, while the hinged lid and front wall were clear Perspex (Figure 1C).

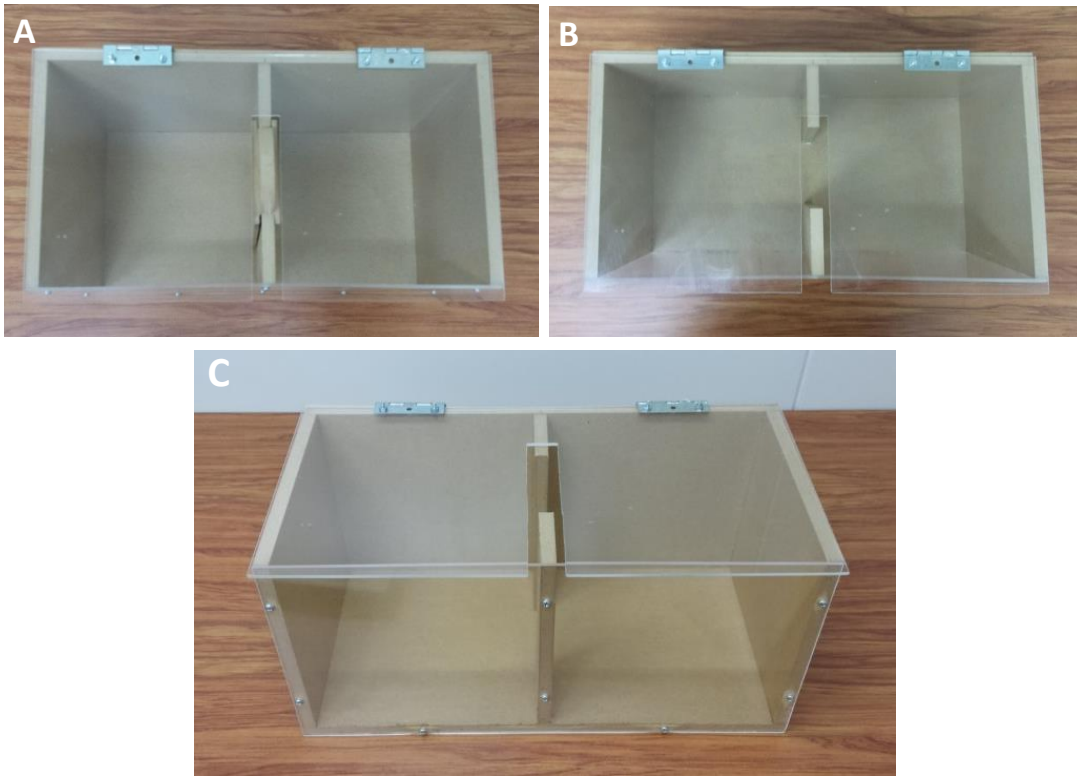

Figure 1. Photographs of the exploration box showing an aerial view (a) with and (b) without the removable guillotine slide, as well as (c) a front view of the apparatus.

Equipment

A small, movable video camera was placed in front of the Perspex wall at the time of retention trials. This was linked to a television set facing perpendicular to the Perspex wall so subjects were unable to be distracted by the screen or the researcher. A computer program, "Mazemovement," was used by the researcher to record the time spent in each half of the exploration box as well as transitions between the novel and familiar halves. The rats' initial

start side was entered and every transition was recorded by pressing the left or right arrows. In this way, the program measured overall time (seconds) in each chamber of the box as well as number of transitions between chambers. Transitions were defined as instances when the subject's head, front paws and shoulders were through the doorway (meaning that the rat would have to move its feet to 'reverse' rather than simply shrink back to the previous half). An auditory "beeper" and earphones were used to indicate 3-second intervals at which the rat's behaviour was recorded. A copy data sheet on which this information was recorded can be found in Appendix 1.

Procedure

Subjects were randomly assigned to experimental groups. To control for possible chamber preference, the acquisition chamber was randomly assigned so that half of the subjects had the left side as their familiar chamber, and half the right side. Each subject was confined to one chamber of the exploration box for 2 hours with the doorway blocked (acquisition trial). After the acquisition trial the subject was removed, intraperitoneally injected with a combination of glucose, caffeine and saline (according to its previously assigned group) and returned to its home cage. Any faecal boli left in the acquisition chamber were removed in order to prevent visual discrimination between the two chambers. 24 hours later, the subject was put back into the same half of the same unwashed exploration box with the guillotine slide removed, and observed for exactly 5 minutes (retention trial). During this trial, the time spent in each half and transitions between the novel and familiar halves were recorded. Also during this trial, every 3 seconds (signalled by an auditory "beeper") the subject's behaviour was recorded on data sheets in terms of the following responses:

1. rearing up on hind legs (R)
2. walking (W)
3. grooming (G)

4. immobile (I)

At the end of the 5-minute session, the number of faecal boli left in the apparatus (defecation) was counted. The apparatus was cleaned thoroughly between subjects with a solution of 4% Powerquat Blue (150 g/l alkylbenzyldimenthylammonium chloride) and allowed to air dry before the next subject's acquisition trial.

Statistical Analysis

Data checking revealed four instances of data entry error (0.002% error rate), which were corrected prior to analysis. A 2x6 (Sex x Group) Analysis of Variance (ANOVA) was used, with planned contrasts comparing drug groups to saline.

Results

As subjects in the saline condition showed a preference for familiarity rather than novelty (as they did in all conditions), the memory criterion was adjusted to accommodate this preference, so that increased time spent in the familiar rather than novel chamber indicated increased memory retention.

Olfactory Memory

A 2x6 (Sex x Group) ANOVA showed a main effect of Sex on time spent in the familiar chamber ($F(1,107) = 10.491$, $p=.002$), but no main effect of Group ($F(5,107) = 1.220$, $p=.305$) and no interaction between Sex and Group ($F(5,107) = .555$, $p=.734$).
The memory performance of males and females was tested separately with a 2-tailed t-test for single samples to determine whether they performed above chance levels (i.e., whether they discriminated between novel and familiar chambers). Females did not perform above chance levels ($t(57)=1.373$, $p=.175$), while males performed significantly above chance levels ($t(60)=5.891$, $p<.001$). Therefore, it was decided to analyse males and females separately in terms of memory.

For males, there was no overall effect of Group on time spent in the familiar half ($F(5,55)=1.676$, $p=.156$), and planned contrasts confirmed that no drug group differed significantly from saline (all t's<1.371, all p's>.203). However, as shown by one-sample t-tests, preference for the familiar chamber was significantly greater than chance (50%) for males receiving saline ($t(9)=2.453$, $p=.037$), glucose ($t(10)=6.272$, $p<.001$), C20+gluc ($t(9)=3.852$, $p=.004$) and C40+gluc ($t(9)=3.757$, $p=.005$). The remaining male groups and all female groups failed to exhibit occupancy behaviour significantly above chance levels (all t's<.396, all p's>.649; see figure 2).

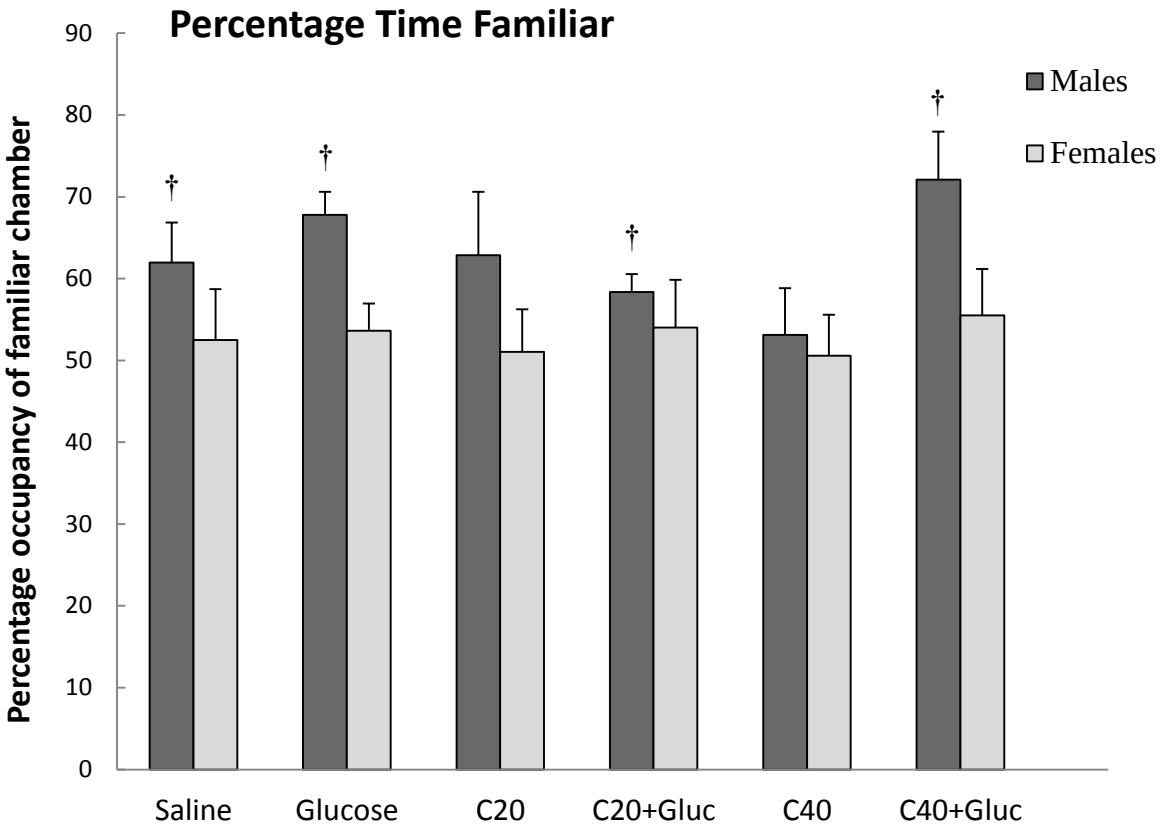

Figure 2: Mean (+S.E.M) percentage time spent in the familiar chamber during retention trials for males and females. Occupancy levels significantly greater than chance expectancy (50%) are indicated (†). No group differed significantly from saline.

2x2 ANOVA

Because of the strong preference of the male control groups, the treatment groups were considered separately in order to determine the relationship between drug effects via a 2x2 (Caffeine x Glucose) ANOVA. For males, the amount of time spent in the familiar chamber was not affected by glucose ($F(1,36) = 1.596$, $p=.215$), or caffeine ($F(1,36) = .120$, $p=.732$) individually, but there was a significant interaction between glucose and caffeine ($F(1,36) = 4.147$, $p=.049$). At the lower dose of caffeine, the presence of glucose had no effect on occupancy, while at the higher dose of caffeine, the presence of glucose significantly improved the discrimination ability (See figure 3). This interaction is supported by t-tests showing that the Caffeine40+Glucose group spent significantly more time in the familiar chamber compared to both Caffeine40 alone ($t(18)=-2.314$, $p=.033$), and Caffeine20+Glucose ($t(18)=-2.185$, $p=.042$), while there was no significant differences between Caffeine20 and Caffeine20+Glucose ($t(18)=.553$, $p=.587$), or Caffeine20 and Caffeine40 ($t(18)=1.009$, $p=.326$).

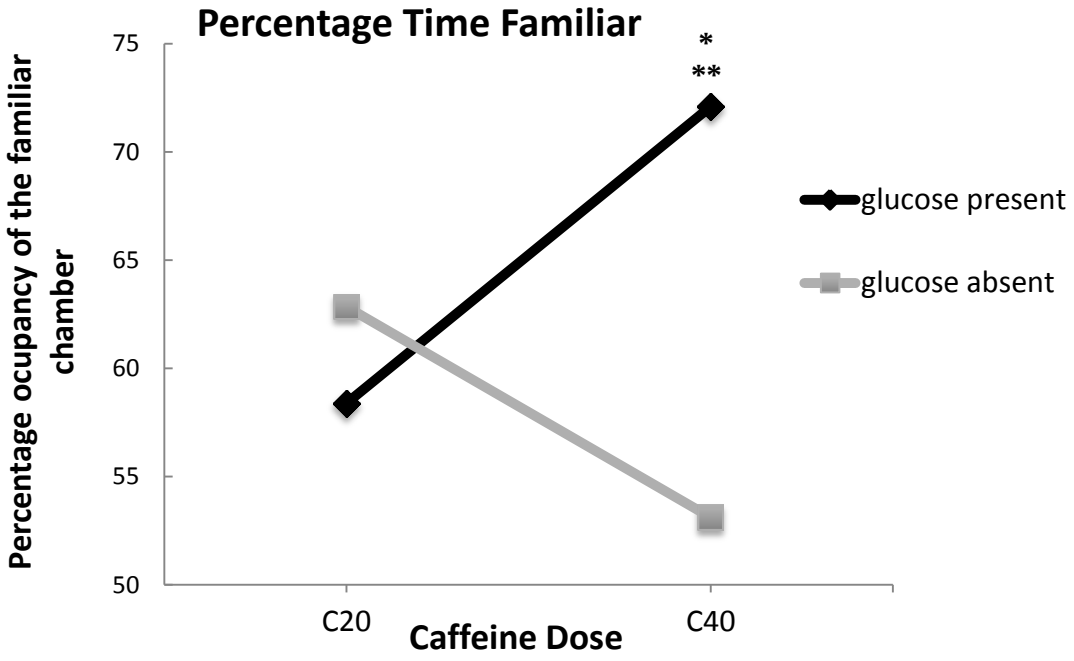

Figure 3: Mean percentage occupancy of the familiar chamber showing an interaction between caffeine dose and the presence or absence of glucose. *C40+Gluc was significantly different to C40, **C40+Gluc was significantly different to C20+Gluc.

Behavioural Results

Male Behaviour

For males, the results for all planned contrasts comparing treatment groups to saline are provided in table 2 below. Separate one-way ANOVAs were conducted to observe the effect of Group on walking, rearing, grooming, immobility, defecation, and transitions.

The administration of caffeine was associated with a reduction in walking behaviour as shown by a main effect of Group on walking ($F(5,55)=4.117$, $p=.003$). Planned contrasts support this, as all caffeine-containing groups spent significantly less time walking compared to saline (see figure 4A).

Similarly, the presence of caffeine was associated with a reduction in rearing behaviour, as supported by a significant effect of Group on rearing ($F(5,55)=7.462$, $p<.001$). Male rats receiving Caffeine40 and Caffeine40+Glucose exhibited significantly less rearing behaviour than saline, while the remaining groups did not (see figure 4B).

There was no main effect of Group on transitions for males ($F(5,55)=1.563$, $p=.186$), however, planned comparisons revealed Caffeine40 +Glucose exhibited marginally significantly fewer transitions than saline ($p=.054$; see figure 4C).

Grooming behaviour was relatively consistent across groups, except for a significant increase in grooming in the Caffeine40+Glucose group (see figure 5A), leading to a main effect of Group on grooming that approached significance ($F(5,55)=2.197$, $p=.068$).

Mirroring the walking results, the administration of caffeine was associated with an increase in immobility, as supported by a significant effect of Group on immobile behaviour for males ($F(5,55)=7.725$, $p<.001$). Planned comparisons confirmed that rats receiving Caffeine20, Caffeine40, and Caffeine40+Glucose exhibited significantly more immobility than saline (see figure 5B).

Defecation levels were relatively consistent across groups, with no overall effect of Group on defecation ($F(5,55)=1.560$, $p=.187$), and planned comparisons confirmed that no groups exhibited significantly different defecation behaviour to saline (see figure 5C).

Table 2: Planned contrasts for males indicating which groups differed from saline (n=10) on measures of behaviour. Significant *t*-values (*p*<.05) are indicated by an asterisk, with *p*-values in brackets. *T*-values approaching significance are italicized.

	Glucose (n = 11)	**C20** (n = 10)	**C20+Gluc** (n = 10)	**C40** (n = 10)	**C40+Gluc** (n = 10)
Walking	-.787 (.435)	**-2.666* (.010)**	**-2.173* (.034)**	**-2.469* (.017)**	**-3.983* (.000)**
Rearing	.549 (.585)	-1.016 (.314)	.381 (.705)	**-3.647* (.001)**	**-3.520* (.001)**
Grooming	-.542 (.590)	-.494 (.623)	-.275 (.785)	-.714 (.478)	**2.087* (.042)**
Immobility	.209 (.835)	**2.416* (.019)**	.875 (.395)	**4.706* (.000)**	**3.725* (.000)**
Defecation	1.135 (.261)	-.403 (.688)	-.403 (.688)	1.614 (.112)	1.109 (.272)
Transitions	-.102 (.919)	-.109 (.913)	.328 (.744)	-1.202 (.235)	***-1.966 (.054)***

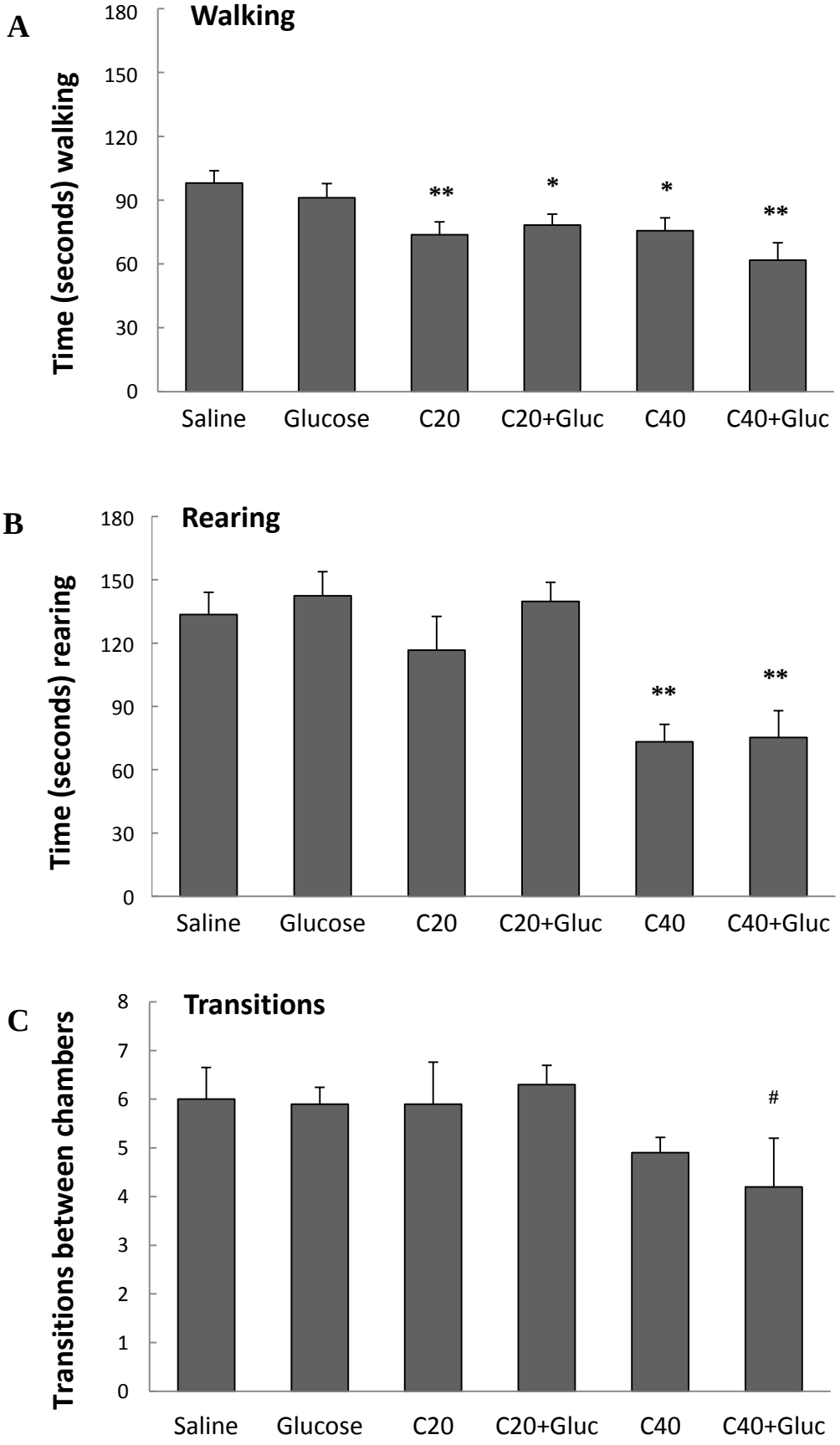

**Figure 4: Male group Mean (+S.E) scores for the exploratory behavioural measures of a) walking, b) rearing, and c) transitions. Significance levels compared to saline are indicated by *****p*<.01, ****p*<.05, and $^{\#}$ *p*<.10.

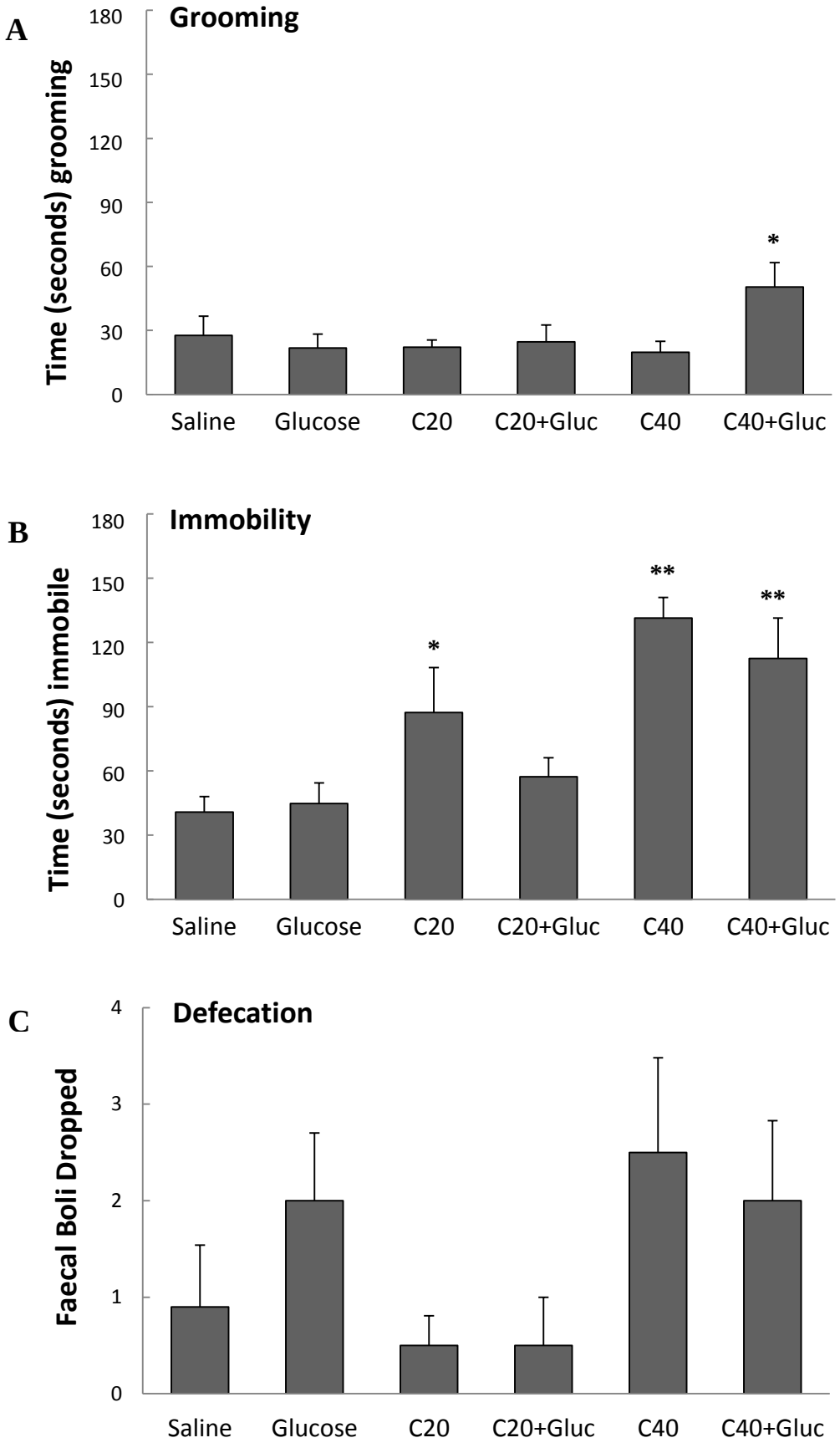

**Figure 5: Male group Mean (+S.E) scores for the behavioural measures of a) grooming, b) immobility, and c) defecation. Significance levels compared to saline are indicated by *****p*<.01, ****p*<.05, and $^{\#}$ *p*<.10.

Female Behaviour

For females, the results for all planned contrasts comparing treatment groups to saline are provided in table 3 below. Separate one-way ANOVAs were also conducted to observe the effect of Group on walking, rearing, grooming, immobility, defecation, and transitions.

Walking behaviour was relatively consistent among groups, as reflected by no overall effect of Group on walking for females ($F(5,52)=2.046$, $p=.087$) . However, Caffeine40 +Glucose spent significantly less time walking compared to saline (see figure 6A).

The administration of caffeine was associated with a reduction in rearing for females, as supported by a significant effect of Group on rearing ($F(5,52)=4.136$, $p=.003$). Females receiving Caffeine40 and Caffeine40+Glucose exhibited significantly less rearing behaviour than those receiving saline, while females in other drug groups did not significantly differ from saline (see figure 6B).

Females showed little variance in transitions between chambers, as supported by no main effect of Group ($F(5,52)=.885$, $p=.498$). Planned comparisons confirmed this, demonstrating that no groups exhibited significantly different behaviour to saline (see figure 6C).

There was no overall effect of Group on grooming behaviour for females ($F(5,52)=1.594$, $p=.178$). However planned comparisons revealed that females receiving Caffeine20 exhibited significantly more grooming behaviour than those receiving saline while Caffeine20+glucose and Caffeine40+glucose were approaching significance (see figure 7A).

Immobility was consistent among females ($F(5,52)=1.758$, $p=.138$). No female groups exhibited significantly different immobility to saline, although caffeine40+glucose was approaching significance (see figure 7B).

Defecation levels were also consistent among females, with no main effect of Group on defecation ($F(5,52)=1.654$, $p=.162$). Planned comparisons confirmed this by showing that no groups exhibited significantly different behaviour to saline (see figure 7C).

Table 3: Planned contrasts for females indicating which groups differed from saline (n=9) on measures of behaviour. Significant *t*-values ($p<.05$) are indicated by an asterisk, with *p*-values in brackets. *T*-values approaching significance are italicized.

	Glucose (n = 9)	**C20** (n = 10)	**C20+Gluc** (n = 10)	**C40** (n = 10)	**C40+Gluc** (n = 10)
Walking	-.259 (.796)	-.471 (.639)	-.369 (.714)	.828 (.411)	**-2.181* (.034)**
Rearing	1.255 (.215)	-1.535 (.131)	-1.130 (.263)	**-2.455* (.017)**	**-2.345* (.023)**
Grooming	.318 (.751)	**2.205* (.032)**	***1.788*** ***(.080)***	1.470 (.148)	***1.862*** ***(.068)***
Immobility	-1.088 (.281)	.107 (.915)	-.011 (.991)	.727 (.470)	***1.703*** ***(.095***
Defecation	-.675 (.503)	-1.385 (.172)	1.073 (.288)	1.108 (.273)	-.450 (.655)
Transitions	.605 (.548)	.424 (.673)	.052 (.959)	.052 (.959)	-1.252 (.216)

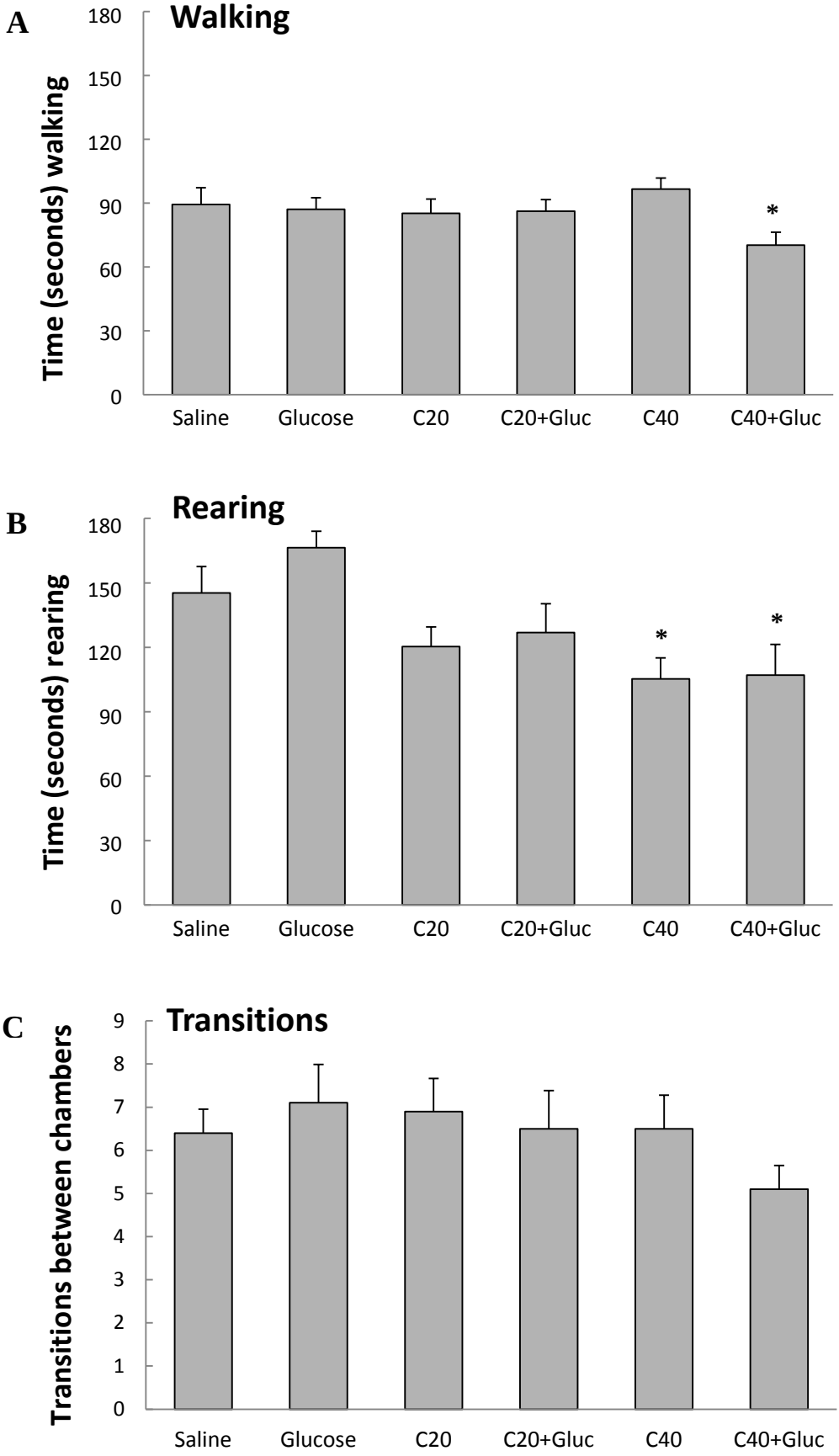

**Figure 6: Female group Mean (+S.E) scores for the exploratory behavioural measures of a) walking, b) rearing, and c) transitions. Significance levels compared to saline are indicated by *****p*<.01, ****p*<.05, and $^{\#}$ *p*<.10.

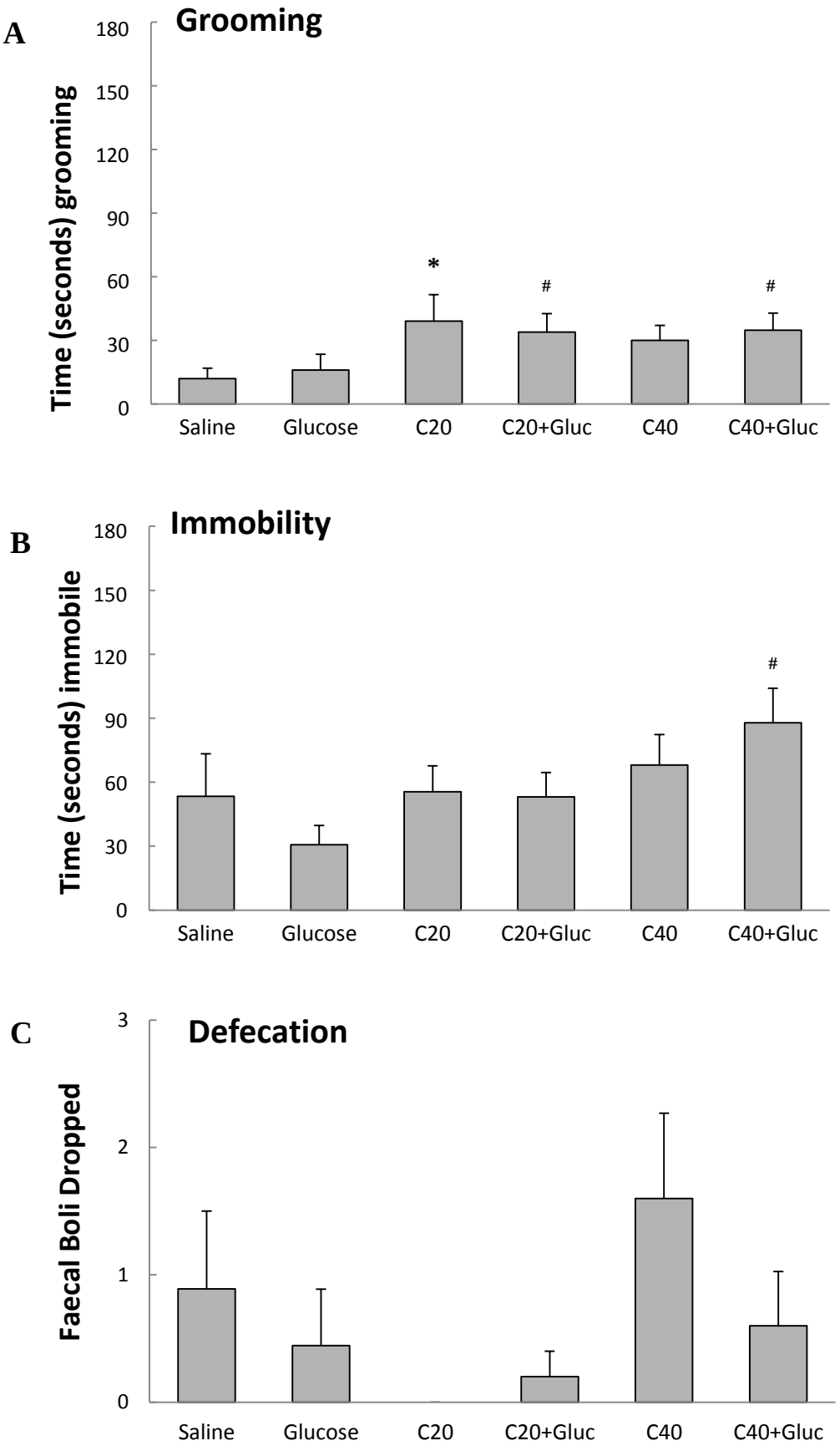

Figure 7: Female group Mean (+SE) scores for the behavioural measures of a) grooming, b) immobility, and c) defecation. Significance levels compared to saline are indicated by **p<.01, *p<.05, and $^{\#}p$<.10

Discussion

Summary of Results

The memory criterion was adjusted to accommodate a preference for familiarity rather than novelty, as all groups spent more than 50% of their time in the familiar chamber. Males spent significantly more time in the familiar chamber compared to both females and chance levels. For males, due to the strong performance of the saline condition, no group differed significantly from saline; however, rats in all glucose-containing groups (Glucose, Caffeine20+Glucose and Caffeine40+Glucose) exhibited an above-chance preference for familiarity. Females showed no discrimination ability in any treatment condition. Behavioural results in the context of emotionality are considered below. Because of the strong preference of the male control groups, the treatment groups were considered separately in order to determine the relationship between substances. While there was no effect of caffeine or glucose alone, an interaction was observed whereby the presence of glucose moderated the relationship between caffeine dose and familiarity preference. At the lower dose of caffeine, the presence of glucose had no effect on occupancy, while at the higher dose of caffeine, the presence of glucose significantly improved discrimination ability.

Preference

It was hypothesised that subjects in this paradigm would have a preference for novelty, due to previous research outcomes (e.g., Hughes, 2006). However, females showed no preference in any condition, while males showed an overall preference for familiarity. This would suggest an inability for females to discriminate between environments, indicating a possible impairment in memory processes, while the familiarity preference in males indicates an effective discrimination between locations. This preference, although unexpected, is not entirely unprecedented.

Previous literature provides ample evidence in favour of rats' preference for novelty over familiarity in a variety of instances including object-preference (Gaskin et al., 2010), place-preference (Klebaur & Bardo, 1999), gustatory-preference (Rowe, Spreekmeester, Meaney, Quirion, & Rochford, 1998) and social-preference (Cavigelli, Michael, West, & Klein, 2011). However, an early experiment exploring odour preferences found that while mature male rats preferred a cage mate's odour to the subject's own odour ($p<.08$), they exhibited a significant preference for their own odour over no odour at all (Carr, Yee, Gable, & Marasco, 1976). Hughes (1991) ran a set of experiments observing the role of olfactory cues in an exploration box devoid of discriminable visual cues. When both halves of the box had been washed, there was no preference. Consistent with Carr et al. (1976), when the novel chamber contained no odour and the familiar chamber contained self-odour, subjects exhibited a significant preference for familiarity. However, when the novel chamber contained cues from other rats, this was consistently preferred over a familiar chamber containing either self-, self- and other-odour, or no odour. Therefore, Hughes concluded that a rat's natural preference for novelty may be interfered with by their attraction to olfactory cues when odour is present in only one chamber. More recently, Prediger, Fernandes, and Takahashi (2005) found that when healthy young rats were given free choice between a chamber with fresh sawdust (unfamiliar) and unchanged sawdust (familiar) they spent significantly more time in the familiar compartment. A separate study found the same familiarity preference for 3- and 6-month-olds, but 12- and 18-month-olds showed no distinct preference for either chamber (Prediger, Batista, & Takahashi, 2005). An acute treatment of caffeine (at 3, 10, or 30mg/kg) reversed this age-related deficit, with subjects spending significantly more time in the familiar compartment. Taken together, these findings provide a basis for the familiarity preference observed in the current study.

Behavioural Analysis

As the male subjects spent significantly more time in the familiar chamber compared to their female counterparts, it is important to consider whether this 'preference' was simply an expression of their emotive state at the time of testing. Rodent behaviour has been extensively studied in a variety of conflict anxiety paradigms; situations where the rodent's inherent avoidance behaviour towards open/exposed areas or a novel object competes with their natural explorative drive (e.g., light-dark box, elevated plus-maze, elevated T-maze, open field test; Donner & Lowry, 2013). While high doses of caffeine have been found to induce anxiety in rats (Braun, Skelton, Vorhees & Williams, 2011; Jain, Hirani, & Chopde, 2005), lower doses have been consistently used without any anxiogenic effects, and some researchers have reported anxiolytic effects for doses up to 50mg/kg (Garcia, Cardenas, & Morato, 2011; Hughes, 2013; Hughes, Hancock, Henwood, & Rapley, 2014; Nadal, Pallares, & Ferré, 1993; Rao, Santos, Paula, Silva, & Campos, 1999; Tang, Kuribara, & Falk, 1989).

In the current study, caffeine was associated with some behavioural differences in males; for example, all caffeine-containing groups exhibited reduced walking compared to saline. Due to the fact that the glucose group had no significant alterations from saline in any measure, it appears that these effects are due to caffeine, or a combination of glucose and caffeine. A dose of 40mg/kg caffeine had the greatest effect on behaviour, especially in combination with glucose, where subjects displayed more grooming and immobility, and less walking, rearing and transitions. Therefore, it appears that the post-acquisition administration of 40mg/kg caffeine in conjunction with 100mg/kg glucose is associated with behavioural differences in a later retention trial. The direction of these behaviours has been previously suggested to represent increased anxiety in other behavioural tests (e.g., Ohl, 2003; Prut & Belzung, 2003), however variations in experimental procedure and apparatus make direct comparisons difficult. Hughes (1991) found that rats exhibited less ambulation and rearing,

and more grooming behaviour when the novel half of an exploration box contained no odour (as in the current paradigm). Rather than an indicator of anxiety, Hughes interpreted these behaviours as a possible response by subjects to insufficient olfactory information, as supported by their tendency to occupy the familiar half in which odour cues were present. This interpretation that increased grooming was not related to anxiety is consistent with the observation that fear (induced by noise and white light) significantly affected rats' heart rates while having no effect on grooming behaviour in an open-field test (Horvath, Kirby, & Smith, 1971). Additionally, in the current study, although the increase in grooming behaviour was significant compared to saline, it represented <17% of the overall behaviour for the Caffeine40+Glucose group. Taken together with the fact that Caffeine40+Glucose had the highest preference for familiarity (M = 72% ± 5.88), a more reasonable interpretation of the behaviour is that these rats had a strong preference for familiarity, which resulted in low exploratory behaviour in a measure of olfactory memory. Therefore, it seems unlikely that the males' preference behaviours can be attributed to pharmacologically-induced anxiety, although future studies may benefit from the inclusion of a more specific anxiety measure in order for this to be conclusively ruled out.

The question remains as to whether males were generally more anxious than females, leading to a familiarity preference. In rodent studies, males generally exhibit more anxious behaviour than females in a variety of conflict anxiety tests, including the light-dark box (Ramos et al., 2002; Voikar, Koks, Vasar, & Rauvala, 2001), the elevated plus-maze (Estanislau & Morato, 2006; Frye, Petralia, & Rhodes, 2000; Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993), the elevated T-maze (Almeida, Tonkiss, & Galler, 1996; Ramos et al., 2002) and the open field test (Frye et al., 2000). In the current study, males spent less time than females in the novel chamber and made fewer transitions between chambers. However, this difference was not attributable to a restriction of movement as both sexes had

comparable walking behaviour, as well as low levels of defecation (with saline groups of both sexes producing an average of less than one bolus during the entire 5 minute trial). Additionally, as stated above, caffeine, at the doses used in the current study, has previously been linked to a reduction in anxiety. However, the saline groups in the current study did not exhibit more anxious behaviour in any category, compared to any drug group. Taken together with the evidence that rats' natural preference for novelty may be interfered with by their attraction to odour cues in only one chamber (Hughes, 1991), this suggests that the increase of time in the familiar chamber is linked to self-cued olfactory attraction rather than a state of anxiety.

Sex differences

While males displayed an overall preference for the familiar chamber with four of six groups obtaining above-chance results, females did not show a significant preference in any condition. This sex difference could be attributed to anxiety at the time of testing, or some aspect of the task being less suited to females, leading to an inability for females to discriminate between chambers which was not able to be attenuated by caffeine or glucose.

Based on the behavioural measures, it is unlikely that the female subjects were in a state of anxiety during testing. Drug groups varied significantly from saline in only four out of a possible thirty instances. None of the female groups exhibited an above-chance preference for either chamber, defecation levels were low ($M<1$ bolus) and rearing levels and transitions were significantly above males, indicating higher exploration activity.

Ascertaining whether these paradigms are less suited to females is a difficult task because there is a lack of research in this area that includes female subjects. The experiments that most closely relate to the current study used only male subjects (Prediger, Batista, & Takahashi, 2005; Prediger, Fernandes, & Takahashi, 2005). Hughes included both male and female subjects, but while one experiment found a preference for familiarity (1991), another

showed no discernible preference in male or female rats receiving saline or low-dose glucose (2006). Furthermore, in the same study an above-chance preference for the novel chamber was observed in subjects receiving 100mg/kg glucose, which is inconsistent with the current results. Sex differences in olfaction have been widely studied in humans, including olfactory awareness, identification, discrimination, preferences and memory (Havlicek et al. 2008; Herz & Inzlicht, 2002; Lehrner, 1993; Platek, Burch, & Gallup, 2001). However, rat studies in these areas are lacking, with the inclusion of female subjects almost exclusively for the purpose of mating, pregnancy, or maternal behaviours (e.g., Antz-Vaxman & Aron, 1986; Bodo & Rissman, 2007; Brennan, Kaba, & Keverne, 1990; Coria-Avila, Ouimet, Pacheco, Manzo, & Pfaus, 2005; Fukushima, Kawagishi, & Moriizumi, 2009; Gelhaye et al., 2011; Keller, Douhard, Baum, & Bakker, 2006; Lévy, Keller, & Poindron, 2004; Moore, 1981; Pierman, Douhard, & Bakker, 2008).

As described earlier, levels of steroid hormones including estrogen (Broverman et al., 1981) and cortisol (Andreano et al., 2008; Bryant et al., 2011; Burgess & Handa, 1992) have been found to alter memory performance. There is evidence that different neuropeptides modulate male and female rats' olfactory memory for conspecifics; arginine vasopressin is essential for males, while females rely on oxytocin (Insel & Hulihan, 1995). When an oxytocin antagonist is administered, female rats are unable to form olfactory memories for either male or female juvenile conspecifics (Engelmann, Ebner, Wotjak, & Landgraf, 1998). Sex hormones including estrogen alter the expression of oxytocin in the brain (Pfaff, Haldar, & Chung, 1992). In female rats, the estrus phase of a menstrual cycle (peak estrogen) is linked to significant increases in oxytocin (Van Tol, Bolwerk, Liu, & Burbach, 1988), while adult ovariectomy decreases total brain oxytocin levels (Miller, Ozimek, Milner, & Bloom, 1989). Additionally, ageing is a factor that affects estrogen levels, with female rodents experiencing signs of reproductive ageing beginning at 9-12 months of age (Yamaguchi &

Yuri, 2014), categorised by persistent estrus followed by persistent diestrus (low estrogen levels; Chakraborty & Gore, 2004). There is evidence that 12-month-old female rats have significantly reduced numbers of estrogen receptors in several areas of the brain, including the olfactory bulb and the hippocampus (Yamaguchi-Shima & Yuri, 2007), as well as a lower level of circulating estrogen (Yamaguchi & Yuri, 2014). Therefore, while it is impossible to determine estrogen and oxytocin levels in the female subjects based solely on behavioural outcomes, it is reasonable to suggest that some age-related changes in hormone levels may have interfered with the females' ability to form olfactory memories – an inability that was unable to be attenuated by either caffeine or glucose. Additional research into direct links between estrogen and oxytocin on olfactory memory in a non-social paradigm would be extremely beneficial to tests relying on olfactory processes in females.

Intact Male Discrimination

Results from the male saline group pose a problem because any explanation that includes drug effects does not account for the significant differences in saline groups between studies. Prediger, Batista and Takahashi (2005) found that 12-month-old rats receiving saline showed no discrimination ability, and the more caffeine they received, the stronger the preference for familiarity became. Conversely, in the current experiment, 12-month-old rats receiving saline showed a significant preference for familiarity, the lower dose caffeine group performed similarly, and subjects receiving high-dose caffeine (without glucose) showed no discrimination. The main difference between studies was the timing of caffeine; in the experiment by Prediger et al. caffeine was administered 30min prior to task performance, rather than immediately post-acquisition. Therefore, subjects were still under the peak influence of caffeine during their discrimination task. This makes interpretation difficult; it could be that the rats' memory was improved only while caffeine was blocking adenosine receptors, but it could also be that some other pharmacological effect was mediating their

behaviour, such as anxiety. However, this still does not explain the differences in saline groups, where neither group were experiencing drug effects. Prediger and colleagues based their method on an earlier study by Soffié and Lamberty (1988), whose saline group showed an intact preference for familiarity (as in the current study). The only difference between these trials is the age of the rats: Soffié and Lamberty used 6-month-old rats while Prediger et al. used 12-month-old rats. This indicates that the younger rats had intact olfactory memory, while the 12-month-old rats had lost this ability (and were able to regain it in the presence of caffeine). This poses another dilemma as it appears that rats in the current study were still very capable of odour discrimination at 12 months of age. Observations of older subjects (>20 months) indicate inconsistencies in odour memory deficits; with some showing significant impairment (Prediger, De-Mello, & Takahashi, 2006) while others show no impairment (Kraemer & Apfelbach, 2004). Additionally, some reports show significant variability within aged cohorts (Robitsek, Fortin, Koh, Gallagher, & Eichenbaum, 2008). While there was no overall difference in olfactory memory between young and old rats, Robisek et al. found a strong correlation between impairment in spatial memory and an impaired olfactory recollection ability, demonstrating a general cognitive decline in some, but not all, aged subjects. The researchers suggested that the severity of impairment may be variable among individuals. Prediger, Batista and Takahashi (2005) cited unpublished data from their own laboratory showing that 12-month-old Wistar rats had intact olfactory discrimination and behaved similarly to young rats in response to cat odour. They suggested that the age at which olfactory deficits are apparent may be dependent on the nature of the task rather than a fixed time-frame. Therefore, the most likely explanation for the discrepancy in discrimination ability between the current study and that of Prediger et al. is that the rate of memory decline may not be consistent in middle-aged subjects.

Another possible contributing factor is strain differences. The current experiment employed PVG/c hooded rats, which appears to be the first time this strain has been used in this apparatus with either glucose or caffeine; related studies have used either Wistar (Hughes, 1991; Prediger, Batista, & Takahashi, 2005; Prediger, Fernandes, & Takahashi, 2005; Soffié & Lamberty, 1988), or Long-Evans strains (Hughes, 2006). There is evidence that PVG rats display some behavioural differences to other strains. In early behavioural research both male and female PVG/c rats were found to have significantly less ambulation than other Hooded or Wistar strains (Broadhurst, 1958). PVG rats were also found to have less locomotion and less anxiety than Sprague-Dawley rats when tested in an open field and elevated plus maze (Schmitt & Hiemke, 1998). However, chronic mild stress was shown to induce anhedonia equally in inbred (PVG) and outbred Lister hooded rats, as well as Sprague-Dawley and three strains of Wistar (Willner, Moreau, Nielsen, Papp, & Sluzewska, 1996). While numerous studies have considered the role of strain differences in non-cognitive outcomes, the literature on memory is sparse. The only strain comparison including PVG rats found that they were slower to acquire a place learning task based on visual cues compared to a Long-Evans strain. However, the addition of olfactory cues facilitated learning of distant visuospatial information for both strains, a benefit which persisted after the olfactory cue had been removed (Lavenex & Schenk, 1997). This study provides evidence that olfactory information can improve learning in strains including PVG rats, however, strain comparisons including olfactory memory and drug effects are yet to be conducted.

Drug Effects

Although the cause of intact male discrimination is unable to be conclusively determined, the fact remains that no combination of caffeine or glucose was able to increase scores significantly above saline levels. Additionally, no treatment group was able to attenuate the performance deficit seen in females. For females, hormone changes occurring

with age were suggested to account for the impairment in the saline group (see above). However, the inability of glucose to attenuate this deficit is particularly surprising, as its effects on memory are well established. As females are under-represented in many rodent studies it is difficult to accumulate a clear picture of the role of sex in the relationship between these substances and memory. However, it is reasonable to propose, based on the evidence, that the underlying cause of olfactory memory loss in middle-aged females was not a deficit that acute glucose or caffeine was able to improve.

For males, the substance effects were somewhat more complicated. Males in all glucose-containing groups (Glucose, Caffeine20+Glucose and Caffeine40+Glucose) showed intact discrimination ability, however, no group was able to perform significantly above saline levels. In addition to the previously-mentioned interpretation issues arising from the intact saline group, there is concern for a potential ceiling effect with the current testing procedure. Previous use of the apparatus (Hughes, 2006) found that drug administration attenuated the forgetting of the familiar half that occurred in the saline group (as seen by their inability to discriminate between chambers). However, when the saline group already shows a significant preference, as in the case of the current study, it is difficult to determine how much of a 'better' memory can be displayed by more time in the preferred chamber. For example, if a subject spent 100% of their time in the familiar side it would not be considered a display of memory, as the rat had not explored both chambers. Therefore, at least some time must be spent in either side of the box to demonstrate a choice. In that vein, once the rat has displayed an above-chance preference, does more time equate to 'more memory' in a linear fashion? The current paradigm seems to allow for somewhat of a dichotomy of memory response; either the subject can or cannot remember. Therefore, while it may have proven effective to assess cognitive normalisers in cases where control groups have some

impairment, it may be less effective at assessing the ability of a drug to enhance cognition above an intact baseline.

This suggestion is supported by the observation of a significant interaction effect between caffeine and glucose in males when assessed separately from saline. At 20mg/kg of caffeine, the presence of glucose had no effect on retention, while, at 40mg/kg of caffeine, the presence of glucose significantly improved discrimination ability. This relationship was unable to be substantiated due to its lack of significance when compared to control groups. However, its presence is important as it suggests a role for a possible synergistic effect whereby males receiving Caffeine40+Glucose had a significantly higher occupancy of the familiar side compared to Caffeine40 alone (and, non-significantly, to Glucose alone). As there are currently no published animal studies assessing the additive effect of caffeine and glucose on memory, the interpretation of these results are limited. However, the significant 2x2 interaction provides a basis for further investigation into this relationship. In future, the use of a testing procedure with a more linear measure of memory improvement may provide the opportunity for this interaction to be fully explored, without the restriction of possible ceiling effects.

Methodological Considerations

Consistent with previous research, each rat was returned to the same unwashed chamber at the start of the trial phase. Although effective in previous studies (Hughes, 2006), this method may have interfered with results in the current study, as rats had a preference for familiarity. In research by Hughes, the subjects' preference for novelty meant that consistently starting them in the familiar chamber strengthened the validity of the results (i.e., despite starting in the familiar side, the rats receiving caffeine showed a preference for novelty). However, in the current study, subjects were consistently placed back into the side for which they subsequently showed a preference. In repeating this experiment, a

counterbalance of familiar and novel start side may offer a more conclusive interpretation of this preference. Alternatively, the apparatus used by Prediger, Batista and Takahashi (2005) and Soffié and Lamberty (1988) contained a central corridor separating the two chambers, and rats were placed into this corridor perpendicularly to the two openings at the time of trial. In this way, rats had free choice of which chamber to explore first, which would also remove the potential bias.

Careful consideration of the methodology provides no other procedural factors that can account for the unexpected male results. The familiar side was counterbalanced so that any possible influences of light/noise from the direction of the door occurred in both the novel and familiar halves (although the testing room door was not opened during trials and no sudden or intrusive noises were observed). Data analysis confirmed that start side had no influence on the percentage of time spent in the familiar compartment ($t(59) = .034$, $p = .973$). Additionally, rats were not able to see any other subjects, the researcher, or the electronic screens during trials, and no interest in external surroundings was observed. Each exploration box had a lid, minimising the possible effect of external olfactory interference. Therefore, the preference behaviour of subjects in the current study is most likely due to memory for self-odour rather than methodological interference.

Concluding Thoughts

Based on the points discussed above, it appears that 12-month-old female rats were unable to recall which side of a two-chamber exploration box they had been assigned to 24 hours earlier, an impairment which was unable to be attenuated by post-acquisition glucose or caffeine, alone or in combination. Conversely, 12-month-old male rats had intact memory for the exploration box as shown by a significant preference for the familiar half, a preference which was unable to be enhanced by post-acquisition glucose or caffeine, alone or in

combination. Sex differences were postulated to involve natural age-related decline of hormone levels, specifically the loss of estrogen in females which causes a reduction in oxytocin (a neuropeptide thought to be integral to female olfactory memory processes). Additional research into direct links between estrogen and oxytocin on olfactory memory in non-social novelty paradigms would be extremely beneficial to tests relying on olfactory processes in females. Additionally, some concerns were raised over the efficacy of the procedure for detecting memory enhancement over an intact baseline, compared to its previous use in attenuating deficits.

By considering this experiment and the findings in the context of what they are, rather than what they were intended to be, the lack of drug effects are not entirely unexpected. Literature in this area provides evidence for the beneficial effect of caffeine in subjects with impaired memory from both pathological (e.g., Alzheimer's disease, ADHD, Parkinson's disease) and non-pathological causes (ageing, alcohol, sleep deprivation, substance use). However, the effects on healthy, intact subjects are minimal at best, restricted to a specific set of constraints (dose, time, task), and lack re-test reliability. If, as suggested, male rats in the current study displayed the behaviour of young, intact rats, it is not entirely surprising that the enhancing effects of caffeine failed to be observed. On the face of it, these results appear to support previous literature that caffeine may be a cognitive normaliser, but not a cognitive enhancer. However, the evidence of an interaction effect between caffeine and glucose in male subjects warrants further research into this relationship with methods that are more suited to intact subjects.

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Appendix 1

Behaviour Data Sheet

Rat/ID_____ Group_____ Sex_____

1)				
2)				
3)				
4)				
5)				
6)				
7)				
8)				
9)				
10)		***2.5 min***		
11)				
12)				
13)				
14)				
15)				
16)				
17)				
18)				
19)				
20) ***1 min***	40) ***2 min***	60) ***3 min***	80) ***4 min***	100) ***5 min***

Transitions = _____ Rearing = _____

Centre squares = _____ Grooming = _____

Corner squares = _____ Immobile = _____

Walking = _____ Faecal boluses = _____